526 Rec'd PCT/PTO 26 MAY 2000

Form PTO-1390 (REV 12-29-99) U.S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	
0652.2080000/REF	
U.S. APPLICATION NO. (IF KNOWN, SEE 37 C.F.R. § 1.5)	
To be assigned 09/555211	
INTERNATIONAL APPLICATION NO	INTERNATIONAL FILING DATE
PCT/EP98/07682	27 November 1998
PRIORITY DATE CLAIMED	
28 November 1997	

OIPE MAY 26 2000 PATENT & TRADEMARK OFFICE 2800

TITLE OF INVENTION

Method for Measuring the Apoptosis

APPLICANT(S) FOR DO/EO/US

STEINLEIN, Peter; HOFFMANN, Johannes; LAMM, Gabor; CHRISTOFORI, Gerhard

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☒ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ Amendments to the claims of the International application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 372(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 C.F.R. 1.97 and 1.98.

12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 C.F.R. 3.28 and 3.31 is included.

13. ☐ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.

14. ☐ A substitute specification.

15. ☐ A change of power of attorney and/or address letter.

16. ☒ Other items or information:
    English translation of Form PCT/RO/101, and
    37 C.F.R. § 1.136(a)(3) Authorization to Treat a Reply As Incorporating An Extension of Time (in duplicate).

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U.S. APPLICATION NO (if known, see 37 C.F.R. 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
To be assigned 09/555211	PCT/EP98/07682	0652.2080000/REF

17. ☒ The following fees are submitted:

	CALCULATIONS PTO USE ONLY	
Basic National Fee (37 CFR 1.492(a)(1)-(5)): Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$970.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$840.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$690.00**		
International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$670.00**		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) ..... **$ 96.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 840.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$ 130.00	

Claims	Number Filed	Number Extra	Rate		
Total Claims	22 - 20 =	2	X **$18.00**	$ 36.00	
Independent Claims	1 - 3 =	0	X **$78.00**	$ 00.00	
Multiple dependent claim(s) (if applicable)			+ **$260.00**	$ 260.00	
			TOTAL OF ABOVE CALCULATIONS =	$ 1266.00	
Reduction of ½ for filing by small entity, if applicable. A Small Entity Statement must be filed. (Note 37 CFR 1.9, 1.27, 1.28)				$	
			SUBTOTAL =	$ 1266.00	
Processing fee of $130.00 for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)).			+	$	
			TOTAL NATIONAL FEE =	$ 1266.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property			+	$	
			TOTAL FEES ENCLOSED =	$ 1266.00	
			Amount to be: refunded:	$	
			charged:	$	

a. ☒ A check in the amount of $ 1,266.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of $ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 19-0036. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit Under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:
STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.
1100 New York Avenue, NW, Suite 600
Washington, D.C. 20005-3934

Raz J Fleshner
SIGNATURE

Raz E. Fleshner
NAME

34,331
REGISTRATION NUMBER

PATENT & TRADEMARK OFFICE DEC 27 2000 JC91

Certificate Under 37 C.F.R. § 3.73(b)

BI Case No. 14/042,44-US
Atty. Docket No. 0652.2080000/REF

Applicants: Peter STEINLEIN, Johannes, HOFFMANN, Gabor LAMM, and Gerhard CHRISTOFORI

U.S. Appl. No.: 09/555,211 which is the U.S. national phase of PCT/EP98/07682 I.A. Filed: Nov. 27, 1998

Entitled: Method for Measuring the Apoptosis

Boehringer Ingelheim International GmbH (Name of Assignee), a Corporation (Type of Assignee, e.g., corporation, partnership, university, government agency, etc.),

states that it is:

1. [X] the assignee of the entire right, title, and interest, or

2. [ ] an assignee of an undivided part interest

in the patent application/patent identified above by virtue of either:

A. [X] An Assignment from the inventor(s) of the patent application/patent identified above. The assignment was recorded in the Patent and Trademark Office at Reel _____, Frame ____, or for which a copy thereof is attached.

OR

B. [ ] A chain of title from the inventor(s) of the patent application/patent identified above to the current assignee as shown below:

1. From: _____ To: _____
The document was recorded in the Patent and Trademark Office at
Reel _____, Frame _____, or for which a copy thereof is attached.

2. From: _____ To: _____
The document was recorded in the Patent and Trademark Office at
Reel _____, Frame _____, or for which a copy thereof is attached.

3. From: _____ To: _____
The document was recorded in the Patent and Trademark Office at
Reel _____, Frame _____, or for which a copy thereof is attached.

[ ] Additional documents in the chain of title are listed on a supplemental sheet.

[X] Copies of assignments or other documents in the chain of title are attached.

[**NOTE:** A separate copy (*i.e.*, the original assignment document or a true copy of the original document) must be submitted to Assignment Division in accordance with 37 CFR Part 3, if the assignment is to be recorded in the records of the PTO. See MPEP 302-302.8]

The undersigned (whose title is supplied below) is empowered to act on behalf of the assignee.

Date: ✓ Dec. 13, 2000

Name: ✓ Dr. Dieter LAUDIEN

Title: ✓ Head of Patent Division

Signature: ✓ Laudien

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SKOF Rev.3/31/00 mac

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#11
3

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	
Steinlein *et al.*	Art Unit: To be assigned
Appl. No.: 09/555,211	Examiner: To be assigned
I.A. Filing Date: November 27, 1998	Atty. Docket: 0652.2080000/REF
For: **Process for Measuring the Apoptosis**	

Preliminary Amendment

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

In advance of prosecution, please amend the application as follows:

Amendments

In the Specification:

At page 1, after the title, please insert the following sentence:

--This application is a 35 U.S.C. § 371 national stage application of the international application, PCT/EP98/07682, filed November 27, 1998, which claims priority benefit to German application no. 197 52 922.4 filed November 28, 1997 and German application no. 198 05 229.4, filed on February 10, 1998, the full disclosures of which are herein incorporated by reference.--

At page 11, line 12, change the word "new" to --neu--.

At page 14, line 6, after the words "For this purpose" please insert --DNA coding for--.

At page 19, line 6, after the word "width" please delete --and pulse width--.

In the Claims:

Please cancel claims 1-18.

Please add the following new claims:

--19. A method of determining the proportion of apoptotic cells in a culture, comprising:

(A) transiently transfecting a population of mammalian cells with a plasmid containing a sequence of interest;

(B) co-transfecting the population of cells with a plasmid containing DNA coding for a flourescent marker protein;

(C) culturing the cells in a suitable nutrient medium so that the DNA sequence of interest or its expressed polypeptide exerts its potential activity on the apoptosis of the cell;

(D) harvesting the cells from (C) and fixing and permeabilizing the cells so that any flourescent protein expressed remains in the cells, while the apoptotic DNA fragments diffuse out;

(E) measuring the proportion of apoptotic cells by measuring total DNA content remaining subsequent to (D);

(F) also, measuring the proportion of transfected cells by quantitating the flourescent marker protein contained in the cells harvested in (D); and

(G) comparing the values obtained in (E) and (F);

thereby, determining the proportion of apoptotic cells in the transfected population.

20. The method of claim 19, wherein the transient transfection method used in (A) and (B) is a receptor-mediated endocytosis method using polyethyleneimine and inactivated Adenovirus.

21. The method of claim 19, wherein the flourescent marker protein in (B) is Green Flourescent Protein.

22. The method of claim 19, wherein the proportion of apoptotic cells are measured in (E) with a DNA binding stain.

23. The method of claim 22, wherein the DNA binding stain is propidium iodide.

24. The method of claim 19, wherein the fixing and permeabilization in (D) is achieved with paraformaldehyde and ethanol, respectively.

25. The method of claim 19, wherein the measuring in (E) and (F) is achieved by fluorescence activated throughflow cytometry analysis.

26. A method of determining whether a gene of interest has an effect on apoptosis of cells in a culture, comprising:

(A) transiently transfecting a population of mammalian cells with a plasmid containing a sequence of interest, thereby obtaining population X; and transiently transfecting another population of the same cells with a control plasmid, thereby obtaining a population Y;

(B) co-transfecting the population of cells with a plasmid containing DNA coding for a flourescent marker protein;

(C) culturing the cells in a suitable nutrient medium so that the DNA sequence of interest or its expressed polypeptide exerts its potential activity on the apoptosis of the cell;

(D) harvesting the cells from (C) and fixing and permeabilizing the cells so that any flourescent protein expressed remains in the cells, while the apoptotic DNA fragments diffuse out;

(E) measuring the proportion of apoptotic cells by measuring total DNA content remaining subsequent to (D);

(F) also, measuring the proportion of transfected cells by quantitating the flourescent marker protein contained in the cells harvested in (D);

(G) for each of the populations X and Y, comparing the values obtained in (E) and (F), thereby calculating a proportion of apoptotic cells; and

(H) comparing the calculated proportion of apoptotic cells obtained in (G) for the populations X and Y.

thereby, determining whether the gene of interest affects the proportion of apoptotic cells in the transfected population.

27. The method of claim 26, wherein the mammalian cells in (A) are tumor cells; and wherein the sequence of interest in (A) encodes a dominant negative signal transmission molecule of a receptor for a survival factor particular to a tumor cell.

28. The method of claim 26, wherein the mammalian cells in (A) are tumor cells; and wherein the sequence of interest in (A) encodes a dominant negative receptor for a survival factor particular to a tumor cell.

29. The method of claim 28, wherein the dominant negative receptor is the IGF-1 receptor.

30. The method of claim 28, wherein the dominant negative receptor is the FGF receptor.

31. The method of claim 28, wherein the dominant negative receptor is the PDGF receptor.

32. A method of determining the effect of a test substance on the pro- or anti-apoptotic activity of a gene of interest, comprising:

(A) transiently transfecting two populations of mammalian cells with an identical plasmid containing a sequence of interest;

(B) co-transfecting the population of cells with a plasmid containing DNA coding for a flourescent marker protein;

(C) culturing one population of transfected cells in a suitable nutrient media containing a test substance, thereby obtaining a population X; and incubating the other population of transfected cells in a suitable medium lacking the test substance, thereby obtaining a population Y;

(D) harvesting the cells from (C) and fixing and permeabilizing the cells so that any flourescent protein expressed remains in the cells, while the apoptotic DNA fragments diffuse out;

(E) measuring the proportion of apoptotic cells by measuring total DNA content remaining subsequent to (D);

(F) also, measuring the proportion of transfected cells by quantitating the flourescent marker protein contained in the cells harvested in (D) ;

(G) for each of the populations X and Y, comparing the values obtained in (E) and (F), thereby calculating a proportion of apoptotic cells;

(H) comparing the calculated proportion of apoptotic cells obtained in (G) for the populations X and Y; and

(I) selecting those test substances which exhibit a synergistic activity;

thereby, determining whether the test substance has an effect on the proportion of apoptotic cells transfected with the gene of interest.

33. The method of claim 32, wherein the mammalian cells in (A) are tumor cells; the gene of interest in (A) is a dominant negative version of a signal transmission molecule of a receptor for a survival factor particular to a tumor cell, which causes the inhibition or absence of a survival factor function; and the test substance exhibits activity synergistic with the inhibition or absence of the survival factor function.

34. The method of claim 32, wherein the mammalian cells in (A) are tumor cells; the gene of interest in (A) is a dominant negative version of a receptor for a survival factor particular to a tumor cell, which causes the inhibition or absence of a survival factor function; and the test substance exhibits activity synergistic with the inhibition or absence of the survival factor function.

35. The method of claim 34, wherein the receptor for a survival factor is the IGF-1 receptor.

36. The method of claim 34, wherein the receptor for a survival factor is the FGF receptor.

37. The method of claim 34, wherein the receptor for a survival factor is the PDGF receptor.

38. The method of claim 32, wherein the test substance in (C) acts synergistically with chemotherapy.

39. The method of claim 33, wherein the test substance in (C) acts synergistically with chemotherapy.

40. The method of claim 34, wherein the test substance in (C) acts synergistically with chemotherapy.

41. A kit for determining the proportion of apoptotic cells in a culture which comprises a carrier means being compartmentalized to receive in close confinement one or more container means wherein:

(A) the first container holds one or more components sufficient for transfection;

(B) another container holds a plasmid containing the sequence coding for the flourescent marker protein;

(C) another container holds an empty vector for inserting the DNA sequence of interest and for control measurements;

(D) another container holds the primary fixing solution;

(E) another container holds the secondary fixing/permeabilizing solution;

(F) another container holds washing solution(s); and

(G) a final container holds a DNA-binding stain.

42. The kit in claim 41, wherein the transfection components in (A) are sufficient to achieve receptor-mediated endocytosis using polyethyleneimine and psoralen/UV-inactivated Adenovirus.

43. The kit in claim 41, wherein the flourescent marker protein in (B) is Green Flourescent Protein.

44. The kit in claim 41, wherein the primary fixing solution in step (D) is 2% paraformaldehyde and the secondary fixing/permeabilization solution in (E) is 70% ethanol.

45. A method of expression cloning of genes which modulate apoptosis comprising:

(A) transiently transfecting a complete cDNA expression library into cells;

(B) co-transfecting the population of cells with a plasmid containing DNA coding for a flourescent marker protein;

(C) culturing the cells in a suitable nutrient medium so that the DNA sequence of interest or its expressed polypeptide exerts its potential activity on the apoptosis of the cell;

(D) harvesting the cells from (C) and fixing and permeabilizing the cells so that any flourescent protein expressed remains in the cells, while the apoptotic DNA fragments diffuse out;

(E) measuring the proportion of apoptotic cells by measuring total DNA content remaining subsequent to (D);

(F) also, measuring the proportion of transfected cells by quantitating the flourescent marker protein contained in the cells harvested in (D);

(G) using FACS sorting to isolate single cells which deviate from an apoptosis background which is to be determined;

(H) isolating, amplifying and selecting the transfected plasmids in a further transfection process; and

(I) characterizing the corresponding genes on the plasmids isolated and amplified in (H) by sequencing and conducting expression and function studies.--

Remarks

Upon entry of the foregoing amendment, claims 19-45 are pending in the application, with claims 19, 26, 32, 41, and 45 being independent claims. Claims 1-18 are sought to be canceled without prejudice or disclaimer of the subject matter therein.

The amendment to the specification on page 11, line 12, replacing the word "new"with the "neu" does not add any new matter. The amendment is to correct a typographical error in the name of the receptor. The support for this change can be found in the title of the reference (# 32 listed on page 25 of the specification, Peles, E. and Yarden, Y. Bioessays 15: 815-24 (1993)) which refers to the gene - "Ne*u* and its ligands: from an oncogene to neural factors." (emphasis added) The abstract of this article states: "The neu gene (also called erbB-2 and

HER-2) encodes ... a receptor...." Therefore, the reference to a "new" gene is an obvious typographical error.

The amendment to the specification on page 14, line 6, in which after the words "For this purpose," the words "DNA coding for" are inserted, introduces no new matter. This phrase is supported on page 2 of the application, in which the method of the invention is described to include transient transfection of "a plasmid containing a DNA sequence of interest" (line 14-15) which corresponds to "*DNA coding for* a dominant-negative version of the human IGF-1 receptor. . . ." Additionally, the proposed phrase is inherent in the sentence and adds no new matter because a dominant-negative version of the human IGF-1 receptor could not be transiently transfected, while DNA coding for it could be.

The amendment to the specification on page 19, line 6, in which the words "and pulse width" after the first occurrence of the word "width" are deleted, does not add any new matter. This phrase is repeated in the sentence to be amended. Therefore the amendment is a correction of a typographical error and is obvious.

New claims 19-45 are sought to be added. Support for new claims 19-45 can be found in the original claims and throughout the specification. In particular, support for claim 19 can be found, *inter alia*, on page 2, lines 8-35, through page 3, lines 1-14, and also in original claim 1. Support for claim 20 can be found, *inter alia*, on page 6, lines 35-36, through page 7, lines 1-2, and also in original claim 2. Support for claim 21 can be found, *inter alia*, on page 4, lines 9-10, and also in original claim 3. Support for claim 22 can be found, *inter alia*, on page 5, lines 10-11, and also in original claim 4. Support for claim 23 can be found, *inter alia*, on page 5, line 11, and also in original claim 5. Support for claim 24 can be found, *inter alia*, on page 8, line 27-29, and also in original claim 8. Support for claim 25 can be found, *inter alia*, on page 4,

lines 20-22, and also in original claim 9. Support for claim 26 can be found, *inter alia*, in Examples 1, 2 and 3. Support for claim 27 can be found, *inter alia*, on page 11, lines 8-10, and page 13, lines 23-27 and in original claim 15. Support for claim 28 can be found on page 11, lines 8-10, and page 13, line 7, and also in original claim 15. Support for claim 29 can be found, *inter alia*, on page 11, line 10, and in original claim 16. Support for claim 30 can be found, *inter alia*, on page 11, line 10, and in original claim 17. Support for claim 31 can be found, *inter alia,* on page 11, line 11, and in original claim 18. Support for claim 32 can be found, *inter alia*, on page 10, lines 12-15, and in original claim 15. Support for claim 33 can be found, *inter alia*, on page 10, lines 12-15, page 13, lines 23-32, and in original claim 15. Support for claim 34 can be found on page 10, lines 12-15, page 13, lines 23-32, and in original claim 15. Support for claim 35 can be found, *inter alia*, on page 10, line 22, and in original claim 16. Support for claim 36 can be found, *inter alia*, on page 10, line 22, and in original claim 17. Support for claim 37 can be found, *inter alia,* on page 10, line 23, and in original claim 18. Support for claim 38 can be found, *inter alia*, on page 10, lines 15-17. Support for claim 39 can be found, *inter alia*, on page 10, lines 15-17. Support for claim 40 can be found, *inter alia*, on page 10, lines 15-17. Support for claim 41 can be found, *inter alia*, on page 16, lines 29-35 through page 17, lines 1-12, and in original claim 10. Support for claim 42 can be found, *inter alia*, on page 17, lines 14-16, and in original claim 11. Support for claim 43 can be found, *inter alia*, on page 4, lines 9-10, and in original claim 12. Support for claim 44 can be found *inter alia*, on page 8, lines 31-33, on page 18, lines 15-17, and in original claim 13. Support for claim 45 can be found on page 15, lines 6-22. These changes are believed to introduce no new matter, and their entry is respectfully requested.

It is not believed that extensions of time are required beyond those that may otherwise be provided for in accompanying documents. However, if additional extensions of time are necessary to prevent abandonment of this application, then such extensions of time are hereby petitioned under 37 C.F.R. § 1.136(a), and any fees required therefor are hereby authorized to be charged to our Deposit Account No. 19-0036.

Respectfully submitted,

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.

Raz E. Fleshner
Attorney for Applicants
Registration No. 34,331

Date: August 2, 2000

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SKGF Rev. 3/4/98

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

#4

In re application of:	
Steinlein *et al.*	Art Unit: To be assigned
Appl. No. 09/555,211	Examiner: To be assigned
Filed: November 27, 1998	Atty. Docket: 0652.2080000/REF
For: **Process for Measuring the Apoptosis**	

Letter to PTO Draftsman: Submission of Formal Drawings

Commissioner for Patents
Washington, D.C. 20231

Sir:

Submitted herewith are three sheets of formal drawings with Figures 1A, 1B, and 2, corresponding to the informal drawings submitted with the above-captioned application. The application number, first-named inventor, and attorney docket number appear on the back of each sheet. Acknowledgment of the receipt, approval, and entry of these formal drawings into this application is respectfully requested.

It is not believed that an extension of time is required, other than any already provided herewith. However, if an extension of time is needed to prevent abandonment of the application, then such extension of time is hereby petitioned. The U.S. Patent and Trademark Office is hereby authorized to charge any fee deficiency, or credit any overpayment, to our Deposit Account No. 19-0036. A duplicate copy of this Letter is enclosed.

Respectfully submitted,

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.

Raz E. Fleshner
Attorney for Applicants
Registration No. 34,331

Date: August 2, 2000

1100 New York Avenue, N.W.
Suite 600
Washington, D.C. 20005-3934
(202) 371-2600

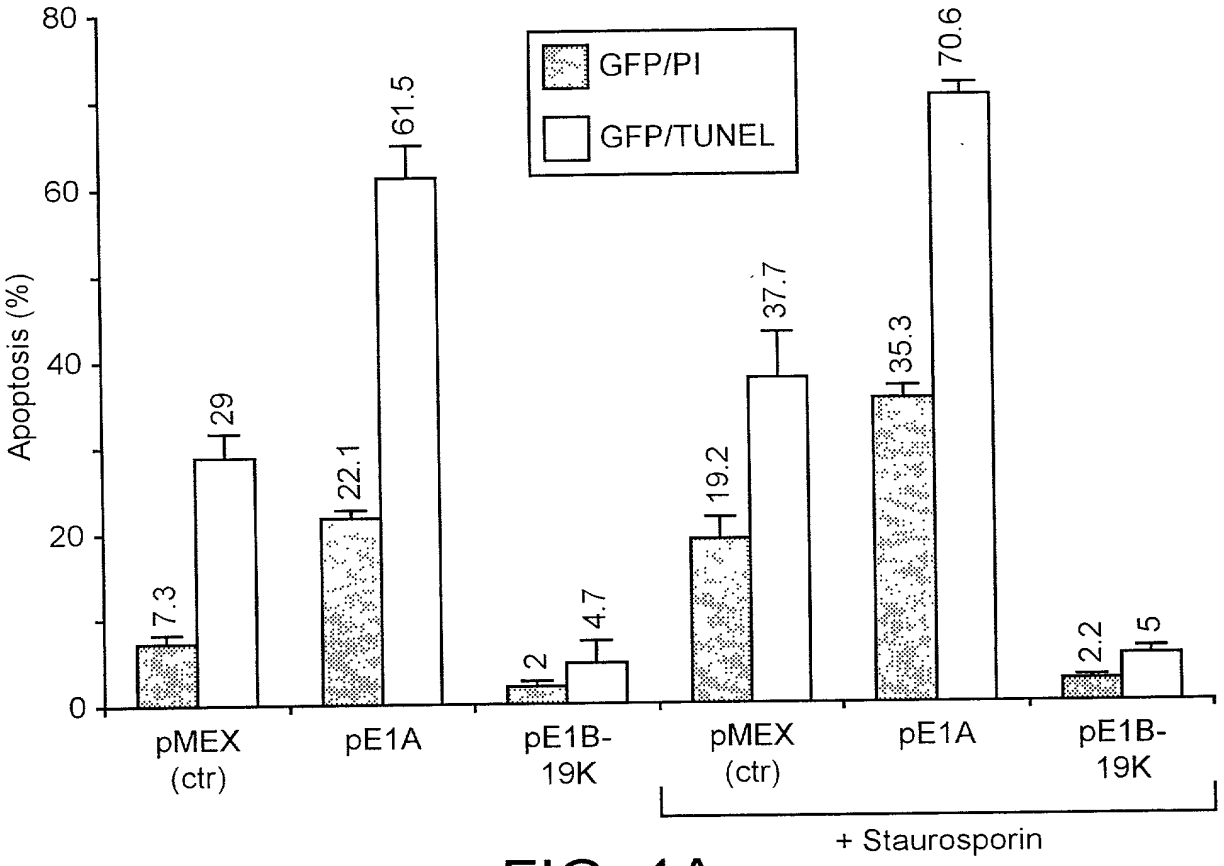
80
60
40
20
0
Apoptosis (%)
GFP/PI
GFP/TUNEL
7.3
29
22.1
61.5
2
4.7
19.2
37.7
35.3
70.6
2.2
5
pMEX (ctr)
pE1A
pE1B-19K
pMEX (ctr)
pE1A
pE1B-19K
+ Staurosporin

FIG. 1A

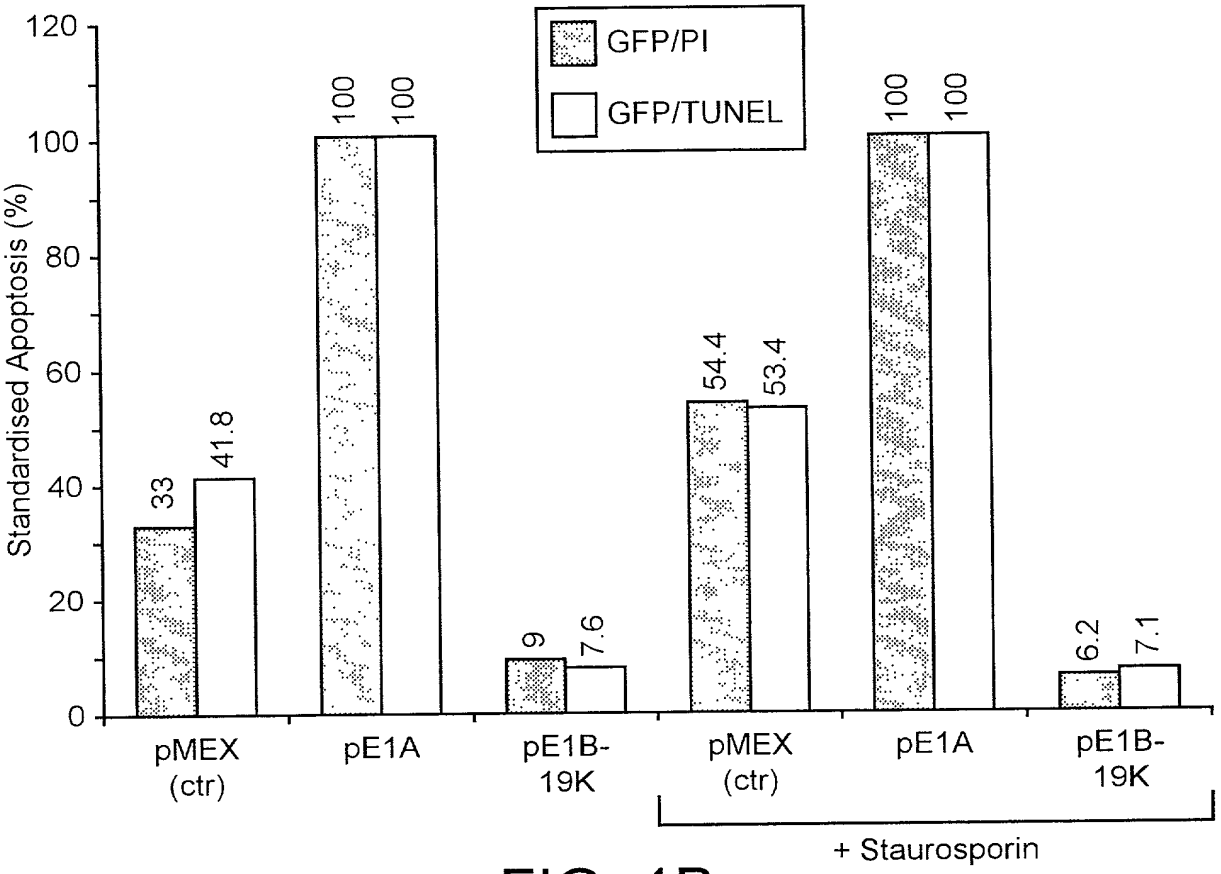
GFP/PI
GFP/TUNEL
Standardised Apoptosis (%)
120
100
80
60
40
20
0
33
41.8
100
100
9
7.6
54.4
53.4
100
100
6.2
7.1
pMEX (ctr)
pE1A
pE1B-19K
pMEX (ctr)
pE1A
pE1B-19K
+ Staurosporin

FIG. 1B

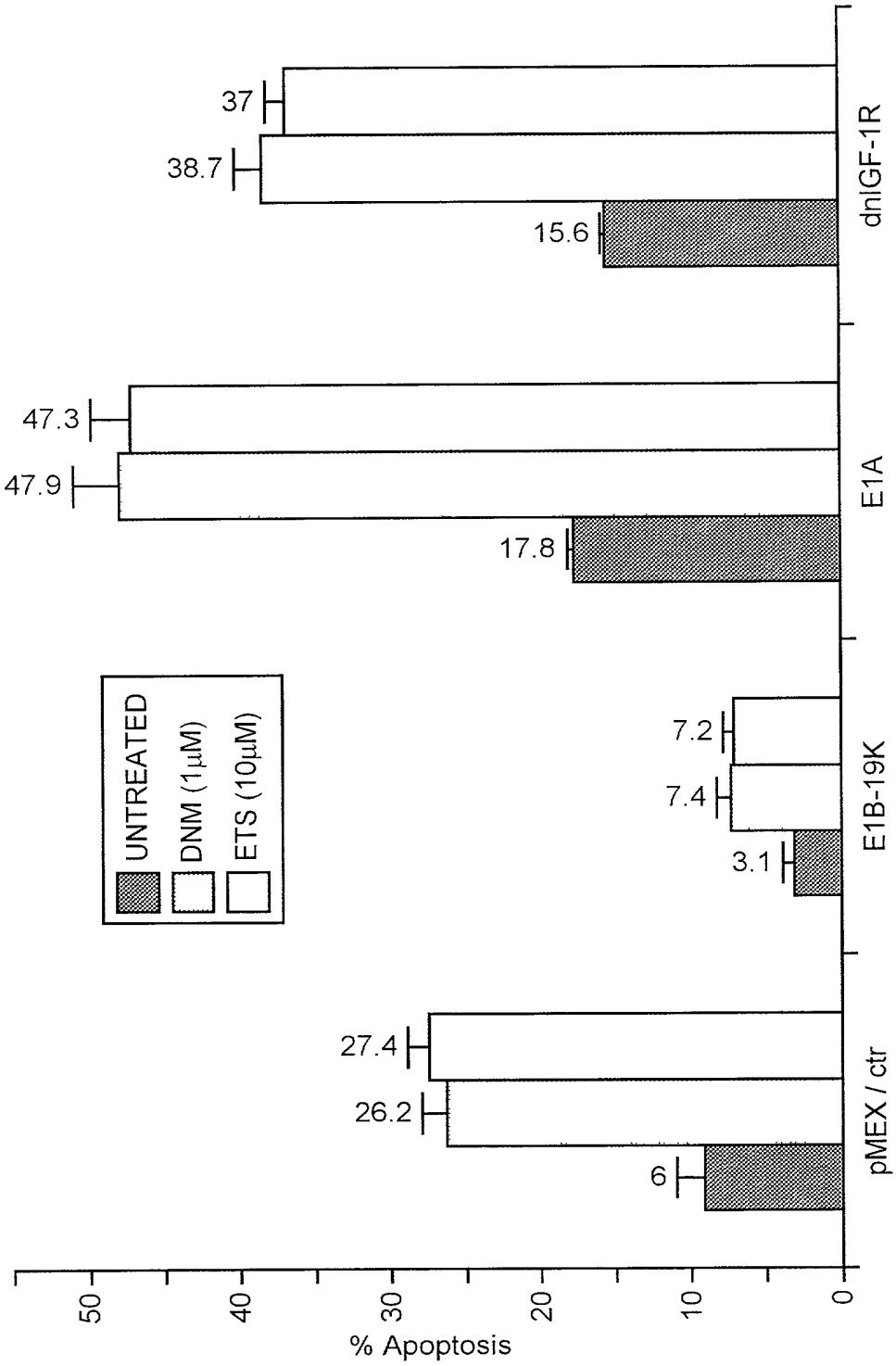
% Apoptosis
0
10
20
30
40
50
pMEX / ctr
6
26.2
27.4
E1B-19K
3.1
7.4
7.2
E1A
17.8
47.9
47.3
dnIGF-1R
15.6
38.7
37
UNTREATED
DNM (1μM)
ETS (10μM)

FIG. 2

3/PART

09/555211

422 Rec'd PCT/PTO 26 MAY 2000

METHOD FOR MEASURING THE APOPTOSIS

The invention relates to the field of biological test methods.

Apoptosis or programmed cell death (PCD) is a genetically controlled cellular suicide mechanism for selectively eliminating unwanted cells (1-4). PCD is an absolutely essential process in a number of biological processes, including embryonic and neural development, the regulation of the immune system, organogenesis, tissue homeostasis and the prevention of diseases such as tumour growth and virus infections. Apoptosis is characterised by blistering of the plasma membrane, shrinkage of the cells, condensation of the nucleus, endonucleolytic cleaving of genomic DNA into fragments of internucleosomal length and the formation of apoptotic bodies.

The methods currently available for investigating apoptosis are based on evaluating morphological changes at the cell level using light, electron or time-lapse microscopy in conjunction with fluorescent vital dyes, the use of annexin V, which can be used to monitor the loss of membrane phospholipid asymmetry during apoptosis (7), or they comprise assays for detecting DNA fragmentation by gel electrophoresis (8) or by *in situ* labelling of DNA strand breaks („nick-end labelling ") (TUNEL) (9).

However, most of these methods are either unsuitable for investigating the effect of genes which play a part in apoptosis, by transient transfection analysis, or,

in the case of the TUNEL method, too expensive and laborious.

The problem of the present invention was to provide a new method of measuring apoptosis which overcomes the disadvantages of the known methods.

This problem was solved by a process for measuring apoptosis which is characterised in that

A) a population of mammalian cells is transiently transfected

ai) with a plasmid containing a DNA sequence of interest which is to be investigated as to whether it or the polypeptide expressed thereby has a pro- or anti-apoptotic activity,

aii) or with a plasmid containing a DNA sequence of interest which is to be investigated as to whether, or by means of which substances, its pro- or anti-apoptotic activity or the activity of the polypeptide expressed thereby can be modulated,

b) and with a plasmid containing a DNA coding for a fluorescent marker protein,

B) in that the cells are incubated in a suitable nutrient medium, optionally in the presence of a test substance, until the DNA sequence of interest or the expressed polypeptide has exerted its potential effect on the apoptosis,

C) in that the cells are harvested and fixed so that the fluorescent protein remains in the cells,

while the DNA fragments formed during apoptosis are able to diffuse out of the cells,

D) in that the proportion of apoptotic cells is determined by measuring the DNA content,

E) in that the proportion of transfected cells is determined by measuring the cells having fluorescent marker protein,

F) and that by comparing the values obtained in steps D and E the proportion of apoptotic cells in the transfected subpopulation of the cells is determined.

The expression "DNA sequence of interest" (hereinafter also referred to as "apoptosis test gene") covers all DNA sequences which affect apoptosis directly or indirectly, as such or in the form of their translated products. Examples of genes which stimulate apoptosis are p53, bax, E1A, examples of apoptosis-inhibiting genes are bcl-2, bcl-x, E1B 19K, the latter group also including the so-called survival factors such as insulin-like growth factors (IGFs). Apoptosis genes of this kind and their activity have been described in summarising articles (e.g. 4, 23, 24).

The apoptosis test genes may be known or unknown genes or fragments thereof. By influencing apoptosis is meant both inducing and reinforcing as well as blocking and attenuating apoptosis.

The method according to the invention allows great variation, e.g. in terms of the markers used for determining the transfected cells and for the

apoptosis, in terms of the plasmids and method of transfection used for transfecting the cells.

The method according to the invention has as one of its essential elements a fluorescent marker protein which serves to indicate the transient transfection of the cells.

The preferred marker protein is Green Fluorescent Protein (GFP). GFP mutants, which are tailored for FACS analysis and are suitable for use within the scope of the present invention, are known from the literature. One example of a suitable GFP mutant was described in (10) ("enhanced Green Fluorescent Protein", eGFP); however, within the scope of the present invention, other mutants may also be used which satisfy the condition that they do not influence cell metabolism, they remain located at the intracellular level and they deliver a measurable fluorescence signal, and in particular they are measurable using current methods of fluorescent activated throughflow cytometry (Fluorescent Activated Cell Sorting (FACS)).

Apart from Green Fluorescent Protein (GFP) other fluorescent marker proteins may also be used. Examples include Blue Fluorescent Protein (BFP) (26) and Yellow Fluorescent Protein (YFP) (25). The properties mentioned above for GFP mutants are essential for the suitability of a marker protein for use in the method according to the invention.

Potential marker proteins and the type and quantity of the plasmids coding for them which are to be used in the assay as well as the most suitable transfection method can be tested as follows, for example: the plasmids coding for the marker proteins are transiently

transfected into mammalian cells, appropriately in the same cells and under the same conditions as are to be used for the method according to the invention. The suitability of the transfected marker proteins is determined by series of measurements in which the transfection efficiency and the efficiency of the reproducible measurement are determined by FACS analysis.

The marker used for the apoptosis is a DNA-binding stain, e.g. propidium iodide (PI), which causes a reduction in fluorescence in the apoptotic subpopulation (14-17). This method of detection is based on the principle that the genomic DNA in cells is broken down endonucleolytically during apoptosis. The small DNA fragments diffuse out of the cell; the reduction in the DNA content to less than twice the set of chromosomes ("sub-2N") is an indication of apoptotic cells.

The reduced fluorescence of PI in cells which are undergoing apoptosis results in the appearance of a characteristic fluorescence peak ("sub-2N-peak") in the area of the G_0/G_1 region of the cell cycle.

Instead of propidium iodide, other DNA-binding stains may be used. Examples of suitable stains of this type are commercially available, e.g. DAPI (4',6'-diamidino-2-phenylindole), acridine orange, ethidium bromide. The most suitable stain can be determined by stimulating cells to apoptosis and then determining by FACS or microscopic analysis whether apoptosis can be reproducibly measured with the candidate stain.

One of the advantages of the method according to the invention is that the fluorescence of the marker

protein and the DNA content can be measured simultaneously, preferably by FACS analysis. Suitable equipment is commercially available.

The invention is applicable to all mammalian cells which can be cultivated. It is a routine procedure for anyone skilled in the art to adjust the standard commercial FACS apparatus to different cell types.

For the transfection of the cells with marker gene on the one hand and the gene of interest on the other hand, all vectors which bring about efficient and reproducible expression in mammalian cells are suitable. Some of the numerous vectors available, including those which are commercially obtainable, contain regulatory sequences capable of achieving high expression rates in a number of mammalian cells. Examples include vectors which contain the CMV-(Cytomegalovirus), the SV40-(Simian virus), MSV (Moloney Sarcoma Virus)-promoter or other powerful promoters non-specific to cell type.

Identical or different vectors may be used as carriers for the marker gene and gene of interest; depending on the type of cell it may be advantageous to use vectors with different promoters, in order to avoid competition between the promoters during the transcription.

With regard to the transfection methods the invention is not subject to any restrictions; theoretically, all the methods known for the transient transfection of mammalian cells can be used, e.g. calcium phosphate, commercially obtainable cationic lipids such as lipofectamine or transfectam, methods based on receptor-mediated, Adenovirus-aided endocytosis, as described e.g. in WO 93/07283, for example using

polyethyleneimine and psoralene/UV inactivated Adenovirus, as described in (21). The transfection method can be optimised, using series of tests in which the transfection conditions, type of cell, nutrient medium etc. are varied, by transfecting with a fluorescent marker protein and determining the expression of the protein by FACS analysis. The optimised conditions for the marker protein are used for the co-transfection with the gene of interest.

After the transfection the cells are incubated in a suitable nutrient medium which is adapted to the particular type of cell. The cells may optionally be stimulated to apoptosis, particularly if the apoptosis test gene is to be investigated for any inhibition of apoptosis. Suitable apoptosis stimuli are known from the literature and commercially available; examples include staurosporin, daunomycin and etoposide. The incubation conditions and the suitability of an apoptosis stimulant are determined in preliminary trials. It is essential for the incubation, particularly its duration, that apoptosis has taken place to an extent which enables any change to be measured by measuring equipment, e.g. by FACS analysis.

The fixing step which is carried out after the incubation is essential to carrying out the process according to the invention.

The essential requirement for the fixing is that the conditions are such that the small subgenomic DNA fragments (internucleosomal fragments, i.e. those with a size of about 200 bp or a multiple thereof) occurring on apoptosis are able to diffuse out of the apoptotic cells, but at the same time the fluorescent marker protein remains in the cell. With the methods available

up till now it was not possible to combine these measurements as the demands made on the fixing with respect to measuring the fluorescent marker protein on the one hand and measuring the DNA content of the cells on the other hand were diametrically opposed and therefore seemed to be irreconcilable. The present invention makes it possible for the first time to carry out both measurements in the same cell population using a suitable fixing step.

In order to determine suitable fixing conditions the following procedure is preferably followed: first of all, the optimum fixing conditions for measuring the fluorescence of the marker protein on the one hand (strong fixing) and the optimum fixing conditions for measuring the DNA content of the cells on the other hand (weakest possible fixing) are determined independently of each other. Starting from the conditions with which the maximum measurements are obtained, the fixing conditions are modified in terms of the reagents (fixing reagent, salts, buffer), the concentration thereof and the fixing time in such a way that the efficiency is affected as little as possible when the two measuring operations are carried out simultaneously.

Preferably, the primary fixing is carried out with paraformaldehyde and the subsequent treatment (secondary fixing/permeabilisation) with ethanol; this treatment has proved most suitable in the tests performed. The primary fixing using 1 to 4 % (w/v), particularly 2 % paraformaldehyde takes place in an isotonic buffered saline solution. Standard solutions are suitable, such as 100 mM NaCl, 3 mM $MgCl_2$, 300 mM saccharose as well as standard commercial physiologically acceptable buffers.

Instead of paraformaldehyde it is also possible to use other reagents such as those which are conventionally used, e.g. in immunohistochemistry. Examples of common fixing agents which can be found in the relevant textbooks (27) include formaldehyde or chloroform/acetone.

Instead of ethanol, which has proved particularly suitable under the conditions selected in the tests carried out for the secondary fixing following on from the primary fixing with paraformaldehyde, it is theoretically possible to use other reagents which render the cell membrane slightly permeable, such as detergents, for example.

The transient expression of genes which modulate apoptosis, for example members of the Bcl family or components of the survival factor signal transduction, and the subsequent quantitative analysis of apoptosis using the method according to the invention make it possible to test chemical compounds to see whether they are capable of specifically influencing the function of apoptosis-modulating genes.

The method according to the invention can be automated by a suitable adaptation of apparatus, e.g. the preparation of samples and the FACS analysis, which makes it suitable for carrying out measurements on a large scale, e.g. in High Throughput Screening methods.

The method in this form is used in the identification of pharmaceutically active substances which are able to modulate apoptosis as a function of the expression of certain genes (apoptosis test genes). The gene whose effect on apoptosis is supposed to be modulated by the

test substance is transiently transfected into test cells and the test cells are incubated with a test substance from a range of substances available. The modulating effect of a test substance on the activity of the test gene is measured directly using measuring instruments.

Methods of this kind can be used for the following screening applications:
a) searching for inhibitors of survival factors and their signal transduction, as well as inhibitors of anti-apoptotic gene products in tumour cells; b) searching for chemicals which, synergistically with chemotherapy, inhibit certain survival factors and their signal transduction in tumour cells; c) searching for chemotherapeutic agents which act synergistically with the inhibition of survival factors.

In one embodiment, the method according to the invention is used in a screening process to investigate the effect of survival factors inherent in tumour cells (receptor ligands such as IGF-I, IGF-II, FGFs (Fibroblast Growth Factors), PDGFs (Platelet Derived Growth Factors) on apoptosis, as mediated by the activation of the corresponding receptors by these factors and the subsequent signal cascade. The assay method can be used in a screening in order to modulate the activity of natural, known or possibly yet to be identified survival factors with respect to a tumour therapy in the course of which the apoptosis of tumour cells is to be intensified. The aim of a process of this kind is particularly to identify substances which, with regard to apoptosis, work synergistically with the inhibition of tumour-specific survival factors.

To detect synergistic effects of this kind the following procedure may be used:

Test cells are prepared, starting from tumour cells, in which the survival factor function is inhibited by introducing into the cells, and expressing, DNAs coding for dominant-negative versions of receptors of the survival factors or for dominant-negative signal transmission molecules of such receptors. Examples of receptors are the IGF-1 receptor (29), FGF receptors (30), PDGF receptors (31), receptors of the EGF-growth factors (32; EGF receptor, Her-2/new/ErbB-2, ErbB-3, ErbB-4). Examples of signal transmission molecules are Ras, Raf, phosphoinositol(3)kinase (=PI(3)-kinase), MAP kinases, type B and type C protein kinase, phospholipase C, and also adapter molecules such as Shc, Grb-2 (33; 34; 35; 36; 37).

Suitable receptor mutants are characterised in that the functional domains of the receptor are modified so that the receptor does indeed bind the ligand but this binding no longer results in the activation of the signal cascade. In the case of IGF-1R the modification comprises the complete absence of the receptor kinase domain or a mutation of the ATP binding site (28). Signal transmission molecules can be modified by inactivating the domains needed for transmission of the signal, e.g. the catalytic domain of an enzyme or the protein binding site of an adapter molecule, by mutation.

The mutants provided for use in a screening process are optimised in preliminary tests for their apoptosis-inducing or -increasing activity in test cells (e.g. fibroblast cell lines which have been made factor-dependent by transfection with the appropriate wild-type receptor) by current methods, in series of tests, by transfecting the mutants into the test cells and measuring the extent of the apoptosis of the test cells using the method according to the invention. The particular functional domains of the mutants are further modified, if necessary, by current molecular biological methods, until an optimum inhibition of the wild-type receptor and its subsequent signal transmission and hence a maximum level of apoptosis of the test cells has been achieved.

In the screening, the test cells are incubated with known chemotherapeutic agents or with substances from a pool which are to be investigated for their potential chemotherapeutic activity and the effect on apoptosis is investigated by the method according to the invention. In particular a screening operation of this kind sets out to find any synergistic activity between the inhibition or absence of the survival factor function in tumour cells and known chemotherapeutic agents or potentially chemotherapeutically active substances.

In order to use the screening process to find substances which exhibit synergism with the inhibition of the survival factor function specifically for certain types of tumour, cells derived from different

types of tumour may be used in a parallel screening test, under otherwise identical experimental conditions.

The cells used as control cells for the specificity of the synergistic activity between the absence of the survival factor function and chemotherapy are cells which are naturally lacking the particular survival factor function the inhibition of which is to be investigated in the assay.

However, theoretically, it is also possible to look for substances which increase the activity of apoptosis-inducing or -increasing molecules. Examples include members of the TNF receptor family (TNF receptors, Fas) and molecules of their signal transmission pathways (caspases). The test principle is exactly the same as for the apoptosis-inhibiting survival factors, except that wild-type or constitutively active versions of these apoptosis molecules are expressed in the tumour cells.

Thus, the main focus of the screening is the search for synergisms which will lead to an increase in tumour cell apoptosis. This provides the prerequisite for therapeutic approaches in which, at the same time as the tumour cell survival function is inhibited, the dosage of chemotherapeutic agents can be significantly reduced without affecting the success of the treatment. The reduction in the chemotherapeutic dose has crucial advantages for the patient, as the toxic side-effects of the chemotherapy can thus be greatly reduced.

Within the scope of the present invention, the method according to the invention was used to investigate whether the signal mediated by the survival factor IGF-II which brings about the survival of β-tumour cells is transmitted by the IGF receptor. For this purpose a dominant-negative version of the human IGF-1 receptor (dnIGF-1R), which has an amino acid substitution in the ATP-binding site (28), is transiently co-transfected into wild-type β-tumour cells with a plasmid carrying Green Fluorescence Protein (eGFP). The cells were then incubated with apoptotic stimuli and/or growth factors, harvested, fixed and stained with propidium iodide in order to determine the DNA content. Using Facs analysis, single transfected cells expressing eGFP were detected and in this population the apoptotic cells were identified by their DNA content of less than 2 N. It was found that the transfection with plasmids which code for the dnIGF-1R leads to a dramatic rise in apoptosis both in untreated wild-type β-tumour cells and in such cells treated with daunomycin or etoposide. It was found that dnIGF-1R intensifies the apoptosis in β-tumour cells with almost the same efficiency as the Adenovirus-E1A protein, one of the most powerful apoptosis-inducing gene products there is. Thus, using the assay method according to the invention, it was possible to show that tumour cells react more sensitively to apoptotic stimuli if the IGF-1R signal transmission is interrupted and that, like IGF-II-deficient tumour cells, they exhibit greater sensitivity to chemotherapeutic agents.

Moreover, the method according to the invention can be used to investigate known genes as to whether and to what extent they modulate apoptosis in different types of cells.

Another use for the method according to the invention is the expression cloning of genes which modulate apoptosis. For this, a complete cDNA expression library is transiently transfected into cells. The method according to the invention is capable of measuring the influence of gene expression within 24 to 48 hours. It is therefore possible to analyse and isolate cells while they are still alive. For this purpose the method is modified by using FACS sorting to isolate single cells which deviate from an apoptosis background which is to be determined. The plasmids transfected into these cells are isolated, amplified and selected in further transfection processes. Plasmids which contain an apoptosis-modulating gene are thus isolated. The corresponding genes are then characterised by sequencing and other studies of expression and function.

To validate the method according to the invention, first of all, in Example 1, established tumour cell lines were used which had been transfected on the one hand with a GFP plasmid and on the other hand with a plasmid containing a pro-apoptotic or an anti-apoptotic gene sequence, or a control plasmid. After the transfection the cells were treated, after a period of rest, with an apoptotic stimulus (control cells remained untreated). Then the detached cells were collected and combined with the trypsinised adherent cells, washed and fixed. After being washed the cells were divided up to make it possible to compare the method according to the invention with the conventional

TUNEL method which uses fluorescent Cy5-dCTP (5-amino-propargyl-2'-deoxycytidine 5'-triphosphate coupled to Cy5 fluorescent stain).

It has been found that the method according to the invention reliably detects the expected pro- or anti-apoptotic effect of the gene products and that the addition of the apoptotic stimulus intensifies the effect observed. Although the sensitivity of the method according to the invention under the conditions selected was slightly less than that of the TUNEL method, the standardised apoptosis value, expressed as the ratio of the maximum apoptosis values of the particular assay achieved with the pro-apoptotic gene, was virtually identical. Compared with the TUNEL method the method according to the invention has the advantage of speed, simplicity and cheapness.

The versatility and reliability of the method according to the invention were confirmed by the use of an untransformed rat fibroblast cell line which reacts less to apoptotic stimuli than the established tumour cell lines.

The method according to the invention makes it possible to establish the potential rôle of a gene product in apoptosis rapidly, effectively and reproducibly.

According to another aspect, the invention relates to a kit for carrying out the process simply as a routine procedure.

A kit of this kind will expediently contain the following components in a number of separate containers:

a) one or more components required for the transfection;
b) a plasmid containing the sequence coding for the fluorescent marker protein;
c) an empty vector for inserting the DNA sequence of particular interest and for control measurements;
d) the primary fixing solution, e.g. paraformaldehyde solution;
e) the secondary fixing/permeabilising solution, e.g. 70 % ethanol;
f) washing solution(s);
g) a DNA-binding stain.

Preferably, the kit contains polyethyleneimine and psoralen/UV-inactivated Adenovirus as transfection components.

Example 1

For this Example, established tumour cell lines (βTC and βHC) were used, derived from β-cell tumours (15) in transgenic mice, in which the regulatory region of the insulin gene (Rip) specifically induces the expression of the large T-antigen of Simian virus 40 (Tag) in the β-cells of pancreatic islets (16).

About 80,000 cells were seeded into a 6 cm well in a 6-well culture dish and cultivated in DMEM, supplemented with 10 % FCS (v/v), 2 mM glutamine, 100 International Units of penicillin and 100 µg/ml of streptomycin, until 70 % confluence was achieved. The cells were transfected with 1 µg of a plasmid coding for eGFP ("enhanced GFP"; pEGFP-C1; Clontech) together with 1 µg of a control plasmid (pMEX; (22)), a pCMV plasmid containing the pro-apoptotic Adenovirus gene E1A or a

pCMV plasmid containing the anti-apoptotic Adenovirus gene E1B-19K (17, 18) using 10 µl of LipofectAMINE (GIBCO-BRL) in accordance with the manufacturer's recommendations. After the transfection the cells were left to stand for 16 h in complete medium, then the cells were either left untreated or treated for a further 16 h with an apoptotic stimulus (800 ng/ml of staurosporin; Sigma) (19, 20). 32 h after the transfection the detached cells were combined with trypsinised, adherent cells, washed twice with 4 ml of PBS and fixed at ambient temperature for 30 min (2 % paraformaldehyde, 100 mM NaCl, 300 mM saccharose, 3 mM $MgCl_2$, 1 mM EGTA (ethyleneglycol-bis-(2-aminoethyl)-tetraacetic acid), 10 mM PIPES (piperazine-N_1N^1-bis[z-ethanesulphonic acid]) pH 6.8). Then they were washed twice with 4 ml of PBS and fixed for 14 h in ice-cold 70 % EtOH.

After the fixing, the cells were washed twice with 4 ml of PBS and divided up. One half of the sample was treated with RNase A (Sigma, St. Louis, USA)(50 µg/ml) in PBS for 30 min, washed twice with 4 ml of PBS and, 30 min before the FACS analysis, stained with propidium iodide in PBS (PI; 50 µg/ml; Sigma, St. Louis, USA). The other half of the sample was incubated for 1 hour at 37°C with 50 µl of TdT reaction mixture (terminal deoxynucleotidyl transferase; Boehringer Mannheim; 200 mM potassium cacodylate, 25 mM of Tris-HCl, pH 6.6, 0.25 mg/ml bovine serum albumen, 1 mM $CoCl_2$; 0.25 nmol FluoroLink Cy5AP3-dCTP [Amersham], 12.5 units of TdT), washed twice with 4 ml of PBS, treated with RNase in PBS (50 µg/ml) for 30 min, washed twice with 4 ml HBS (from this step onwards HBS was used instead of PBS because DAPI has a tendency to produce microprecipitates in PBS), stained with DAPI in HBS (10 µg/ml; Sigma) for 20 min and analysed using a

Becton Dickinson FACS Vantage apparatus. The FACS analysis of the PI-stained cells was carried out with a Becton Dickinson FACScan apparatus fitted with a so-called "doublet discrimination module", with which cell aggregates are discriminated by calculating the pulse width and pulse width. The results of the tests are shown in Fig. 1. Fig. 1A shows the number of apoptotic βHC 13T tumour cells (% apoptosis) in the entire eGFP-positive cell population. The black bars indicate the determination of the sub-2N DNA content (GFP/PI); the white bars indicate the incorporation of fluorescent Cy5AP3-dCTP during the TdT reaction (GFP/TUNEL). The addition of staurosporin is shown. An excitation wavelength of 488 nm was used for eGFP and PI, an excitation wavelength of 647 nm was used for Cy5 and UV of a wavelength range of 51 - 364 nm was used for DAPI. The emission fluorescence was collected using a 530/20 nm narrow band filter for eGFP, a 610 nm blocking filter for PI, a 675/20 nm narrow band filter for Cy5 and a 424/44 narrow band filter for DAPI. Doublets were excluded by means of pulsed processing. eGFP-expressing cells were selected and analysed for Cy5- or PI-fluorescence. The data were analysed using CELLQuest software (Becton Dickinson). Each bar represents the average of 3 transfections, standard deviations are indicated by error bars. Each measurement comprised 40,000 total events, selected according to size and single cells. The transfection efficiency was 20-30 %.

Fig. 1B shows the standardised percentage of apoptosis for the various constructs. The apoptosis index was standardised using the following function: (% apoptosis in X/% apoptosis in eGFP-C1/E1A) x 100. The apoptotic index was standardised for each of the detection

methods used and for each subsequent transfection treatment (+/- staurosporin).

Example 2

In this Example an untransformed rat fibroblast cell line designated Rat1A was used. The cells were transiently transfected using either LipofectAMINE, as described in Example 1, or polyethyleneimine (PEI 2000)-DNA-Adenovirus complexes (WO 93/07283). Moreover, regarding the treatment of the cells and determination of apoptosis, using the process according to the invention on the one hand and the TUNEL method on the other hand, the procedure was exactly as described in Example 1. A comparison of the different transfection methods and methods of measuring apoptosis is shown in the Table. Each value represents the average of 3 transfections; the standard deviation is given (s.d.).
The efficiency of the transfection methods was 25-30 %.

Example 3

In this Example wild-type β-tumour cells (15) were used. The dominant-negative IGF-1 receptor construct used, which is under the control of the CMV promoter and in which the codon 1003 in the ATP binding site is mutated from lysine to alanine, was described by (28).
As described in the previous Examples, wild-type β-tumour cells were seeded at a density of 80,000 cells in triplicate in 6-well culture dishes. 24 h later the cells were co-transfected with pEGFP-C1 and a control plasmid (Fig. 2: pMEX/ctr) or with pEGFP-C1 and an expression plasmid, coding either for Adenovirus-E1A

(Fig. 2: E1A), Adenovirus-E1B-19K (Fig. 2: E1B-19K) or the dominant-negative IGF-1R. 36 h after transfection, daunomycin (Fig. 2: shaded bar) or etoposide (Fig. 2: white bar) was added to the culture medium in a concentration of 1 μM or 10 μM. Transfected but untreated cells are indicated in Fig. 2 by black bars. 12 h after treatment with daunomycin or etoposide the cells were harvested, fixed and treated with propidium iodide, as described in the previous Examples. The apoptotic cells were also determined using the methods described above. For each measurement 40,000 events were collected; the transfection efficiency was 25-30 %. The standard deviation is indicated by error bars.

Table

Trans-fected Construct	LipofectAMINE			
	Propidium iodide		TUNEL	
	% apoptosis (s.d.)	% apoptosis standardised	% apoptosis (s.d.)	% apoptosis standardised
pMEX (Ctr)	3.3 (0.7)	68.8	6.5 (0.6)	71.4
pE1B-19K	1.4 (0.3)	29.2	2.2 (0.5)	24.2
pE1A	4.8 (0.4)	100	9.1 (2.6)	100
	PEI / Adeno			
pMEX (Ctr)	1.2 (0.2)	52.2	7.5 (0.3)	52.1
pE1B-19K	0.7 (0.1)	30.4	5.2 (0.8)	36.1
pE1A	2.3 (0.6)	100	14.4 (2.7)	100

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Patent Claims

1) Process for measuring apoptosis, characterised in that

A) a population of mammalian cells is transiently transfected

ai) with a plasmid containing a DNA sequence of interest which is to be investigated as to whether it or the polypeptide expressed thereby has a pro- or anti-apoptotic activity,

aii) or with a plasmid containing a DNA sequence of interest which is to be investigated as to whether, or by means of which substances, its pro- or anti-apoptotic activity or the activity of the polypeptide expressed thereby can be modulated,

b) and with a plasmid containing a DNA coding for a fluorescent marker protein,

B) in that the cells are incubated in a suitable nutrient medium, until the DNA sequence of interest or the expressed polypeptide has exerted its potential activity on the apoptosis,

C) in that the cells are harvested and fixed so that the fluorescent protein remains in the cells, while the DNA fragments formed during apoptosis are able to diffuse out of the cells,

D) in that the proportion of apoptotic cells is determined by measuring the DNA content,

E) in that the proportion of transfected cells is determined by measuring the cells with fluorescent marker protein,

F) and that by comparing the values obtained in steps D and E the proportion of apoptotic cells in the transfected subpopulation of the cells is determined.

2. Process according to claim 1, characterised in that the transfection of the cells is carried out with polyethyleneimine and inactivated Adenovirus.

3. Process according to claim 1, characterised in that the fluorescent polypeptide defined in A b) is Green Fluorescent Protein.

4. Process according to claim 1, characterised in that the DNA content is measured with a DNA-binding stain.

5. Process according to claim 4, characterised in that the stain is propidium iodide.

6. Process according to one of claims 1 to 5, characterised in that the incubation according to step B) is carried out in the presence of a test substance.

7. Process according to one of claims 1 to 6, characterised in that the incubation according to step B) is carried out in the presence of a substance which stimulates apoptosis.

8. Process according to one of claims 1 to 7, characterised in that the primary fixing in step C is carried out with paraformaldehyde and the secondary fixing/permeabilisation of the cells is carried out with ethanol.

9. Process according to one of claims 1 to 8, characterised in that the measurements defined in steps D and E are carried out in one step using fluorescence-activated throughflow cytometry analysis.

10. Kit for performing the process according to claim 1, characterised in that it contains the following components in several separate containers:

a) one or more components required for the transfection;

b) a plasmid containing the sequence coding for the fluorescent marker protein;

c) an empty vector for inserting the DNA sequence of particular interest and for control measurements;

d) the primary fixing solution;

e) the secondary fixing/permeabilising solution;

f) washing solution(s);

g) a DNA-binding stain.

11. Kit according to claim 10, containing as component a) polyethyleneimine and psoralen/UV-inactivated Adenovirus.

12. Kit according to claim 10, containing as component b) a plasmid coding for Green Fluorescent Protein.

13. Kit according to claim 10, containing as component d) an approximately 2% paraformaldehyde solution and as component e) about 70 % ethanol.

14. Use of the process according to claim 1 for identifying substances which modulate the pro- or anti-apoptotic activity of genes or gene products.

15. Use according to claim 14 for identifying therapeutically effective substances which exhibit a synergistic activity with the inhibition or absence of the survival factor function from tumour cells, characterised in that the cells are tumour cells, in that the DNA sequence according to aii) is a dominant-negative version of a receptor for a survival factor particular to a tumour cell or a signal transmission molecule of a receptor of this kind, and in that the cells are incubated in the presence of the test substance.

16. Use according to claim 15, characterised in that the DNA sequence according to aii) is a dominant-negative version of the IGF-1 receptor.

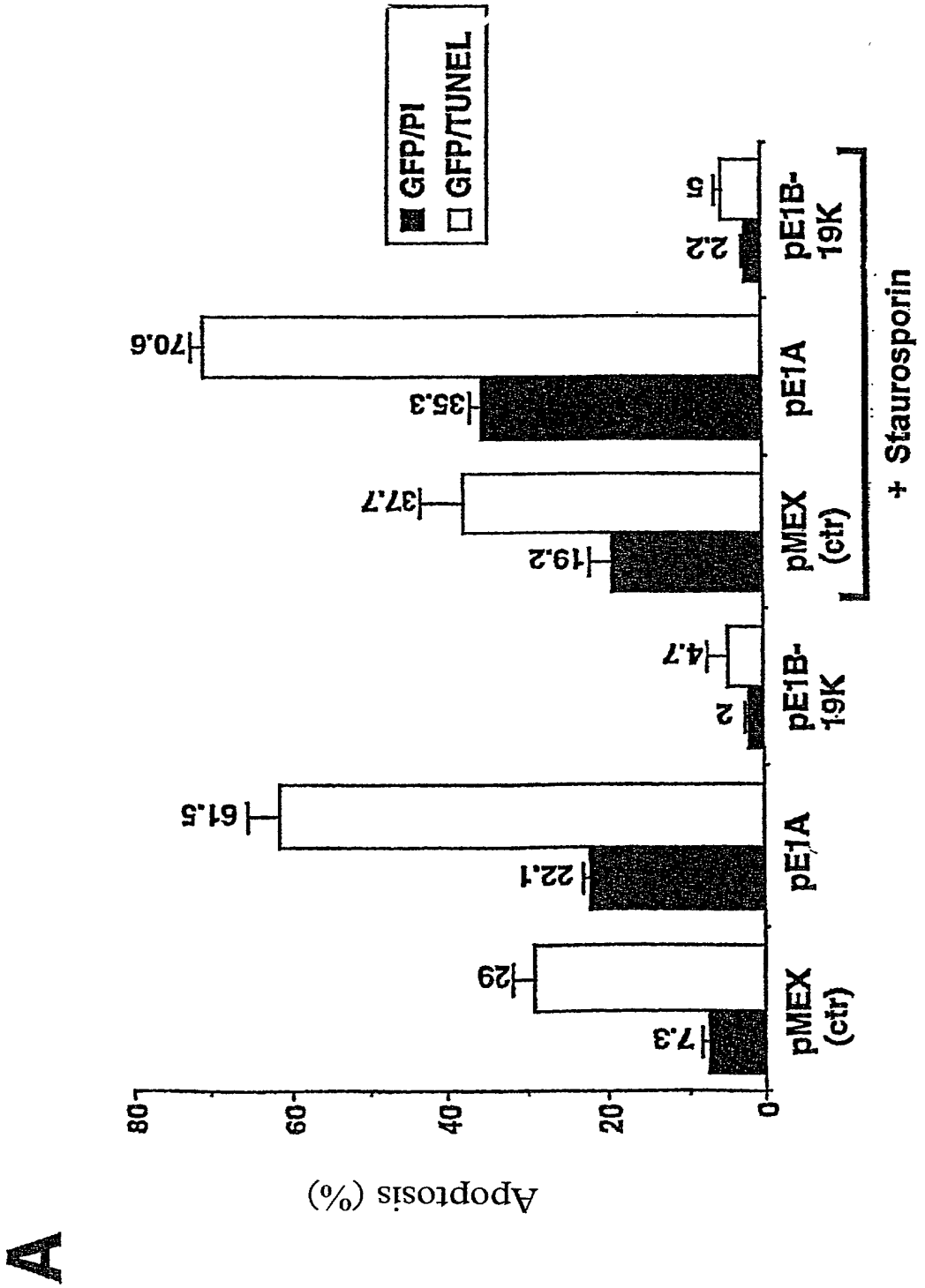
17. Use according to claim 15, characterised in that the DNA sequence according to aii) is a dominant-negative version of an FGF receptor.

18. Use according to claim 15, characterised in that the DNA sequence according to aii) is a dominant-negative version of a PDGF receptor.

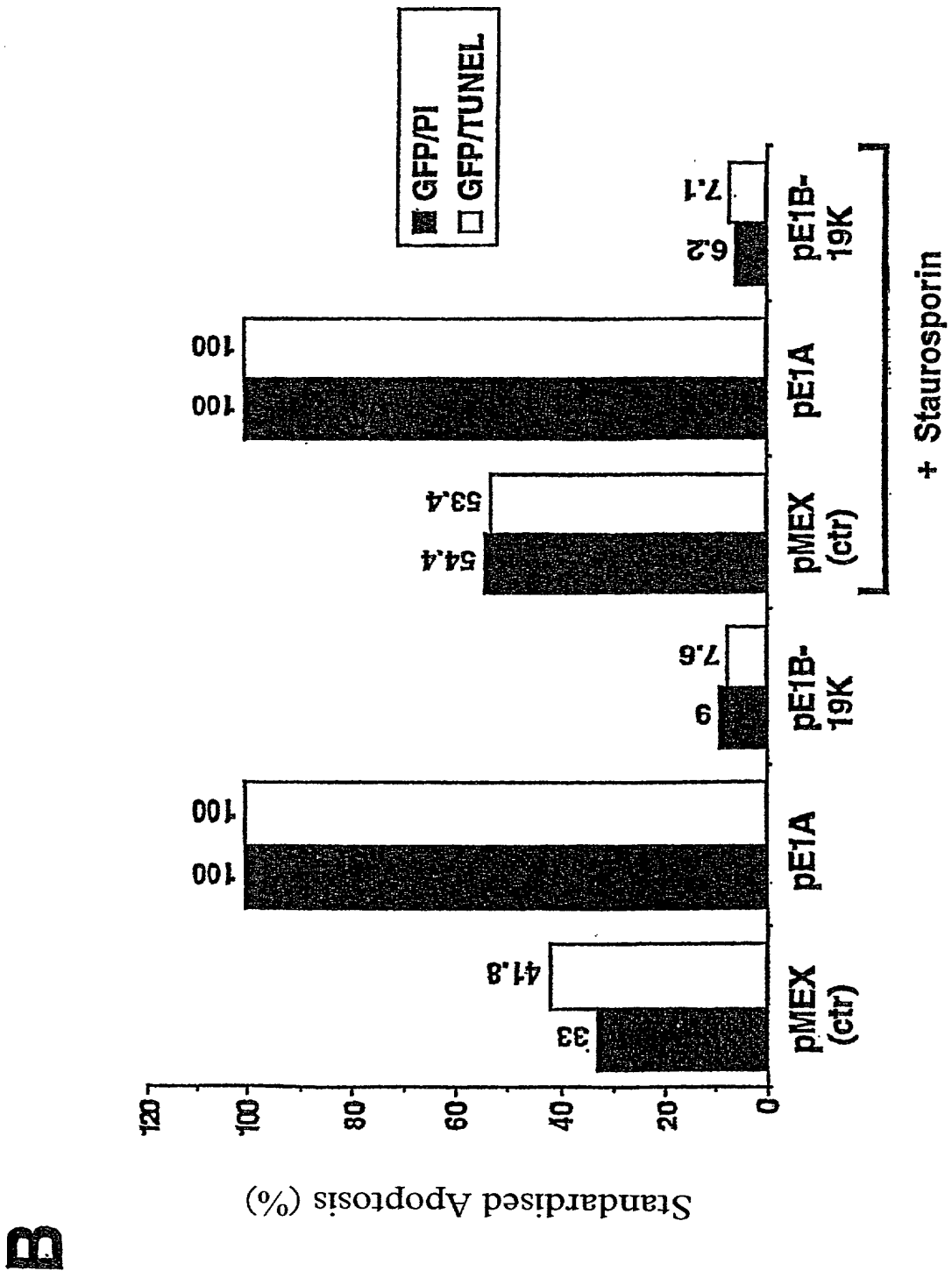
Abstract

Process for measuring apoptosis quickly and easily. Mammalian cells are co-transfected with a plasmid which codes for a fluorescent protein, and with a plasmid carrying a gene of interest. After incubation and gentle fixing, apoptosis is measured by determining the DNA content of the cells using DNA-binding stain and determining the proportion of transfected cells by throughflow cytometry. The process can be used to identify substances which modulate the pro- or anti-apoptotic activity of genes or gene products.

1/3
Fig. 1 A

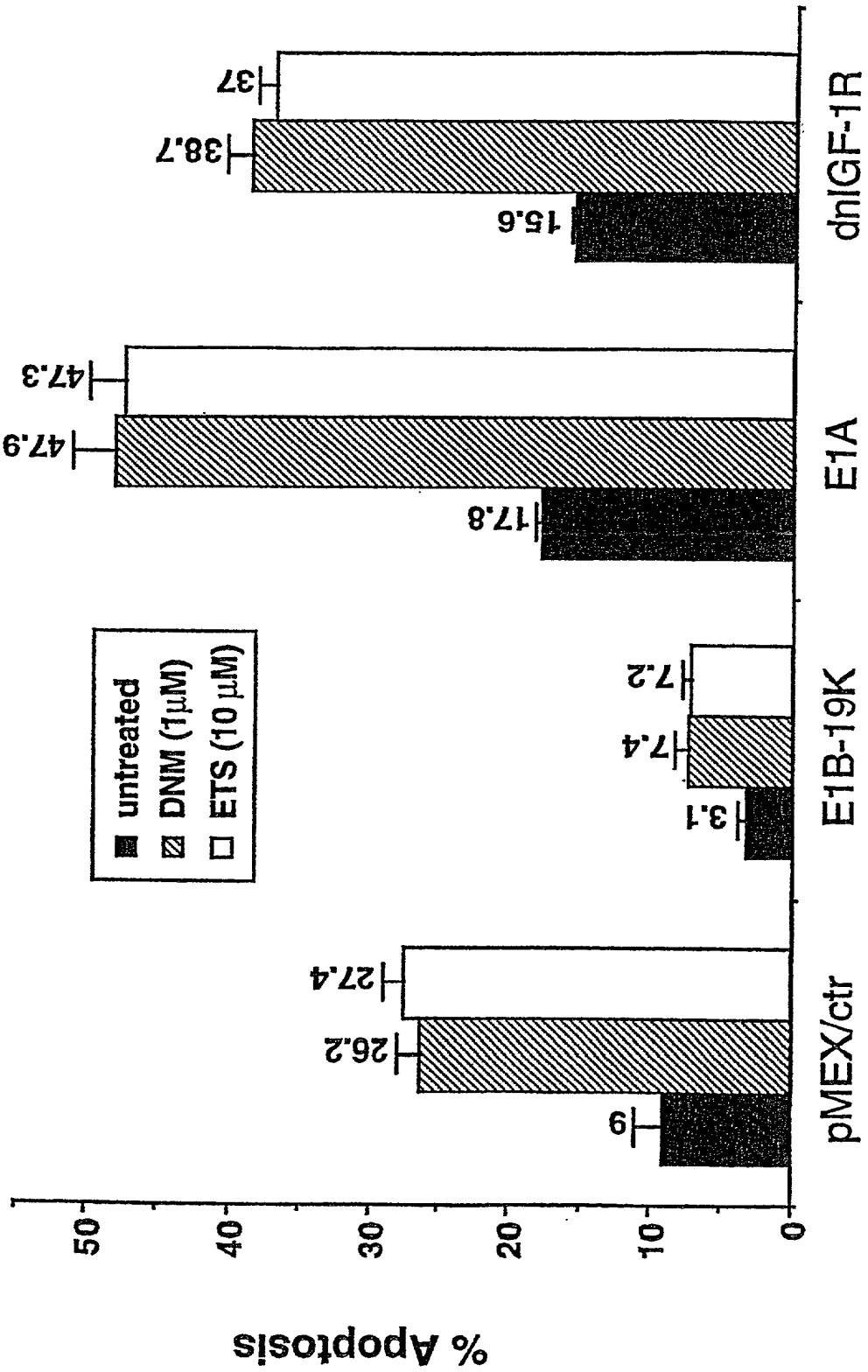

A
Apoptosis (%)
0
20
40
60
80
GFP/PI
GFP/TUNEL
pMEX (ctr)
pE1A
pE1B-19K
+ Staurosporin
7.3
29
22.1
61.5
2
4.7
19.2
37.7
35.3
70.6
2.2
5

2/3
Fig. 1 B


GFP/PI
GFP/TUNEL
Standardised Apoptosis (%)
0
20
40
60
80
100
120
33
41.8
100
100
9
7.6
54.4
53.4
6.2
7.1
pMEX (ctr)
pE1A
pE1B-19K
+ Staurosporin

B

3/3
Fig. 2


% Apoptosis
0
10
20
30
40
50
pMEX/ctr
9
26.2
27.4
E1B-19K
3.1
7.4
7.2
E1A
17.8
47.9
47.3
dnIGF-1R
15.6
38.7
37
untreated
DNM (1μM)
ETS (10 μM)

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JC91

Declaration for Patent Application

BI Case No. 14/042,44-US
Atty. Docket No. 0652.2080000/REF

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter that is claimed and for which a patent is sought on the invention entitled Method for Measuring the Apoptosis, the specification of which is attached hereto unless the following box is checked:

☒ was filed on November 27, 1998 (I.A. Filing Date);
as United States Application Number 09/555,211, which is the U.S. national phase of International Application No. PCT/EP98/07682 ; and
was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information that is material to patentability as defined in 37 C.F.R. § 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT international application, which designated at least one country other than the United States listed below, and have also identified below any foreign application for patent or inventor's certificate, or PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)			Priority Claimed	
197 52 922.4	Germany	28/11/1997	☒ Yes	☐ No
(Application No.)	(Country)	(Day/Month/Year Filed)		
198 05 229.4	Germany	10/02/1998	☒ Yes	☐ No
(Application No.)	(Country)	(Day/Month/Year Filed)		

I hereby claim the benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below.

(Application No.)	(Filing Date)
(Application No.)	(Filing Date)

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s), or under § 365(c) of any PCT international application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose information that is material to patentability as defined in 37 C.F.R. § 1.56 that became available between the filing date of the prior application and the national or PCT international filing date of this application.

PCT/EP98/07682	27 November 1998	Pending
(Application No.)	(Filing Date)	(Status - patented, pending, abandoned)
(Application No.)	(Filing Date)	(Status - patented, pending, abandoned)

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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Signature of second inventor ✓ Joh. Hoffmann	11/17/00 Date ✓
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Citizenship	Austrian
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3-00

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Signature of fifth inventor		Date
Residence		
Citizenship		
Post Office Address		

4-00

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(Supply similar information and signature for subsequent joint inventors, if any)

ASSIGNMENT

U.S. Appl. No. 09/555,211
BI Case No. 14/042,44-US
Atty. Docket No. 0652.2080000/REF

In consideration of the sum of One Dollar ($1.00) or equivalent and other good and valuable consideration paid to each of the undersigned inventor(s): Peter STEINLEIN, Johannes, HOFFMANN, Gabor LAMM, and Gerhard CHRISTOFORI, the undersigned inventor(s) hereby sell(s) and assign(s) to Boehringer Ingelheim International GmbH (the Assignee) his/her entire right, title and interest, including the right to sue for past infringement and to collect for all past, present and future damages:

check applicable box(es) ☒ for the United States of America (as defined in 35 U.S.C. § 100),
☒ and throughout the world,

(a) in the invention(s) known as Method for Measuring the Apoptosis for which application(s) for patent in the United States of America has (have) been executed by the undersigned on ✓11/17/00, 11/17/00, 11/30/00 and 11/23/00 (also known as United States Application No.: 09/555,211 which is the U.S. national phase of International Application No. PCT/EP98/07682, international filing date November 27, 1998) in any and all applications thereon, in any and all Letters Patent(s) therefor, and

Reg. No. 32,893

(b) in any and all applications that claim the benefit of the patent application listed above in part (a), including continuing applications, reissues, extensions, renewals and reexaminations of the patent application or Letters Patent therefor listed above in part (a), to the full extent of the term or terms for which Letters Patents issue, and

(c) in any and all inventions described in the patent application listed above in part (a), and in any and all forms of intellectual and industrial property protection derivable from such patent application, and that are derivable from any and all continuing applications, reissues, extensions, renewals and reexaminations of such patent application, including, without limitation, patents, applications, utility models, inventor's certificates, and designs together with the right to file applications therefor; and including the right to claim the same priority rights from any previously filed applications under the International Agreement for the Protection of Industrial Property, or any other international agreement, or the domestic laws of the country in which any such application is filed, as may be applicable;

all such rights, title and interest to be held and enjoyed by the above-named Assignee, its successors, legal representatives and assigns to the same extent as all such rights, title and interest would have been held and enjoyed by the Assignor had this assignment and sale not been made.

The undersigned inventor(s) agree(s) to execute all papers necessary in connection with the application(s) and any continuing (continuation, divisional, or continuation-in-part), reissue, reexamination or corresponding application(s) thereof and also to execute separate assignments in connection with such application(s) as the Assignee may deem necessary or expedient.

The undersigned inventor(s) agree(s) to execute all papers necessary in connection with any interference or patent enforcement action (judicial or otherwise) related to the application(s) or any continuing (continuation, divisional, or continuation-in-part), reissue or reexamination application(s) thereof and to cooperate with the Assignee in every way possible in obtaining evidence and going forward with such interference or patent enforcement action.

The undersigned inventor(s) hereby represent(s) that he/she has full right to convey the entire interest herein assigned, and that he/she has not executed, and will not execute, any agreement in conflict therewith.

The undersigned inventor(s) hereby grant(s) Robert Greene Sterne, Esquire, Registration No. 28,912; Edward J. Kessler, Esquire, Registration No. 25,688; Jorge A. Goldstein, Esquire, Registration No. 29,021; Samuel

L. Fox, Esquire, Registration No. 30,353; David K.S. Cornwell, Esquire, Registration No. 31,944; Robert W. Esmond, Esquire, Registration No. 32,893; Tracy-Gene G. Durkin, Esquire, Registration No. 32,831; Michele A. Cimbala, Esquire, Registration No. 33,851; Michael B. Ray, Esquire, Registration No. 33,997; Robert E. Sokohl, Esquire, Registration No. 36,013; Eric K. Steffe, Esquire, Registration No. 36,688; Michael Q. Lee, Esquire, Registration No. 35,239; Steven R. Ludwig, Esquire, Registration No. 36,203; Raz E. Fleshner, Esquire, Registration No. ~~34,331~~; John M. Covert, Esquire, Registration No. 38,759; and Linda E. Alcorn, Esquire, Registration No. 39,588; all of STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C., 1100 New York Avenue, N.W., Suite 600, Washington, D.C. 20005-3934, power to insert in this assignment any further identification that may be necessary or desirable in order to comply with the rules of the United States Patent and Trademark Office for recordation of this document.

IN WITNESS WHEREOF, executed by the undersigned inventor(s) on the date opposite his/her name.

Date: ✓ 11/17/00 Signature of Inventor: ✓ P. STEINLEIN
Peter STEINLEIN

Date: ✓ 11/17/00 Signature of Inventor: ✓ Joh. Hoffmann
Johannes HOFFMANN

Date: ✓ 11/30/00 Signature of Inventor: ✓
Gabor LAMM

Date: ✓ 23.11.00 Signature of Inventor: ✓
Gerhard CHRISTOFORI

POWER OF ATTORNEY FROM ASSIGNEE

BI Case No. 14/042,44-US
Atty. Docket No. 0652.2080000/REF

Boehringer Ingelheim International GmbH, a corporation of Germany, having a principal place of business at Binger Straße 173, D-55216 Ingelheim am Rhein, GERMANY, is assignee of the entire right, title and interest for the United States of America (as defined in 35 U.S.C. § 100), by reason of an Assignment to the Assignee executed on ✓ 11/17/00, 11/17/00, 11/30/00 and 11/23/00, respectively of an invention known as Method for Measuring the Apoptosis (Attorney Docket No. 0652.2080000/REF), which is disclosed and claimed in a patent application of the same title by the inventor(s) Peter STEINLEIN, Johannes, HOFFMANN, Gabor LAMM, and Gerhard CHRISTOFORI (said application filed on [international filing date November 27, 1998] at the U.S. Patent and Trademark Office, having Application Number 09/555,211 which is the U.S. national phase of International Application No. PCT/EP98/07682,).

RLE Reg. No. 32,893

The Assignee hereby appoints the following U.S. attorneys to prosecute this application and any continuation, divisional, continuation-in-part, or reissue application thereof, and to transact all business in the U.S. Patent and Trademark Office connected therewith: Robert Greene Sterne, Esq., Reg. No. 28,912; Edward J. Kessler, Esq., Reg. No. 25,688; Jorge A. Goldstein, Esq., Reg. No. ~~29,021;~~ Samuel L. Fox, Esq., Reg. No. 30,353; David K.S. Cornwell, Esq., Reg. No. 31,944; Robert W. Esmond, Esq., Reg. No. 32,893; Tracy-Gene G. Durkin, Esq., Reg. No. 32,831; Michele A. Cimbala, Esq., Reg. No. 33,851; Michael B. Ray, Esq., Reg. No. 33,997; Robert E. Sokohl, Esq., Reg. No. 36,013; Eric K. Steffe, Esq., Reg. No. 36,688, Michael Q. Lee, Esq., Reg. No. 35,239; Steven R. Ludwig, Esq., Reg. No. 36,203; Raz E. Fleshner, Esq., Reg. No. 34,331; John M. Covert, Esq., Reg. No. 38,759; and Linda E. Alcorn, Esq., Reg. No. 39,588. The Assignee hereby grants said attorneys the power to insert on this Power of Attorney any further identification that may be necessary or desirable in order to comply with the rules of the U.S. Patent and Trademark Office.

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SIGNATURE: ✓ ppa. [signature] i.V. [signature]

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TITLE: ✓ Head of Patent Division Authorized Person of Patent Division

DATE: ✓ Dec. 13, 2000

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